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ORIGINAL PAPERS

Interaction and colocalization of PGP9.5 with JAB1 and p27^{Kip1}

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PGP9.5 (UCH-L1) is a member of the ubiquitin C-terminal hydrolase (UCH) family of proteins that is expressed in neuronal tissues. Our previous studies have shown that PGP9.5 was highly expressed in primary lung cancers and lung cancer cell lines. Additionally, the frequency of PGP9.5 over expression increases with tumor stage, indicating that PGP9.5 may play a role in lung cancer tumorigenesis. Here, we used the yeast two-hybrid system to identify proteins that interact with PGP9.5. We show that PGP9.5 interacts with at least three proteins, one of which is JAB1, a Jun activation domain binding protein that can bind to p27^{Kip1} and is involved in the cytoplasmic transportation of p27^{Kip1} for its degradation. Here, we show that PGP9.5 is associated with JAB1 *in vitro* and *in vivo*; and that both proteins can be a part of a heteromeric complex containing p27^{Kip1} in the nucleus in lung cancer cells. Furthermore, under serum-restimulation, nuclear translocation of both PGP9.5 and JAB1 coincides with a reduced level of p27^{Kip1} in the nucleus. In contrast, when cells are contact inhibited, both PGP9.5 and JAB1 became more perinuclear and cytoplasmic in localization while p27^{Kip1} was presented only in the nucleus. Therefore, PGP9.5 may contribute to p27^{Kip1} degradation via its interaction and nuclear translocation with JAB1.
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Introduction

A great number of regulatory proteins are modified by conjugation with ubiquitin (Ub) or ubiquitin-like proteins (Ulp). This modification acts as a targeting signal, delivering the modified protein to different locations in the cell and modifying its activity, half-life, or interaction with other molecules (Ciechanover *et al*., 2000; Hochstrasser, 2000). Ubiquitin modification of proteins plays an important role in many critical pathways including regulation of the cell cycle , modulation of the cellular response to stress and extracellular effectors, morphogenesis of neural networks, DNA repair, regulation of the immune and inflammatory responses, down-regulation of cell surface receptors and ion channels, and biogenesis of organelles (Ciechanover *et al*., 2000). Deubiquitination, the reversal of this modification, is carried out by deubiquitinating enzymes that are thiol proteases. The process of deubiquitination is now being recognized as a participant in the regulatory processes of the cell (Wilkinson, 1997).

We have previously analysed gene expression patterns in non-small cell lung carcinoma (NSCLC) using the serial analysis of gene expression (SAGE) approach and identified PGP9.5 as a protein highly expressed in primary lung cancers and lung cancer cell lines (Hibi *et al*., 1998). PGP9.5 represents one of the members of the ubiquitin hydrolase (UCH) family, UCH-L1. Three human UCH isozymes have been cloned which exhibit marked tissue specificity (Wilkinson, 1997). UCH-L1 was found to be only localized to the brain and testis (Chung and Baek, 1999) and is also thought to play a role in the development of some forms of Parkinson's disease (Wilkinson, 2000). We have observed aberrant PGP9.5 expression in 22 of 24 lung cancer cell lines and in 54% of 98 primary NSCLC cases. Moreover, PGP9.5 expression was strongly associated with advanced stage lung cancers (Hibi *et al*., 1999).

Biochemically, UCH isoforms tend to hydrolyze monoubiquitinated substrates (Larsen *et al*., 1998). These monoubiquitinated substrates are not targeted for degradation by the 26S proteasome pathway, but this reversible modification is being recognized as an important regulatory strategy (Wilkinson, 1997, 2000). Recently, UCH-L3 was shown to function as a terminal hydrolase for Nedd 8, an ubiquitin-like protein and ubiquitin (Wada *et al*., 1988). Because little is known regarding UCH-L1 specific substrates or its biological activity *in vivo*, and our previous results indicated that PGP9.5 may play a role in lung cancer

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tumorigenesis, we used a yeast two-hybrid screen to identify proteins that interact with PGP9.5 in an effort to obtain further insights into the biological function of this protein.

The results presented here demonstrate the unbiased interaction of PGP9.5 with JAB1, a protein initially described as a co-activator of c-*jun* (Claret *et al*., 1996). JAB1 was later shown to be a component of a novel protein complex called signalosome (Seeger *et al*., 1998). Several studies have also shown that JAB1 promotes the phosphorylation and cytoplasmic translocation of p27^{Kip1} for its subsequent degradation in the cytoplasm (Tomoda *et al*., 1999). Our results show that PGP9.5 associates *in vivo* and *in vitro* with JAB1. The two proteins are localized throughout the cells. However, they appeared to be able to form a heteromeric complex that includes p27^{Kip1} and can co-localize to the nucleus of lung cancer cells. Furthermore, the nuclear localization of PGP9.5 and JAB1 coincided with a reduced nuclear staining of p27^{Kip1} during serum restimulation. In contrast, when cells were contact inhibited, both PGP9.5 and JAB1 were redistributed in the cytoplasm while p27^{Kip1} was present only in the nucleus. These observations suggested that PGP9.5 might play a role in lung cancer growth by regulating the level of nuclear JAB1, which can then lead to an increased degradation of p27^{Kip1}.

Results

Identification of proteins interacting with PGP9.5

A yeast two-hybrid screen was used to identify candidate proteins that interact with PGP9.5 in an expression cDNA library derived from fetal brain fused with the Gal4 transactivation domain. The library was screened three times and a total of 1.67×10^7 independent clones were analysed (Table 1). Several specifically interacting cDNA clones were identified that were positive for the expression of the selection markers (histidine and adenine). Four cDNA clones interacted specifically with PGP9.5 upon reconstitution analysis. Subsequent sequence analysis showed that two of the clones had an identical 1.4-kb insert with 100% homology to the human ubiquitin-conjugating enzyme UBC9 (Shen *et al*., 1996). Another clone with a 2.5 kb insert was identical to RanBPM, a Ran associated protein that is present throughout the cells (Nishitani *et al*., 2001). The fourth clone had a 1.05 kb insert identical to a human Jun activation domain-binding protein that was initially called p38^{JAB1} (Claret *et al*., 1996). We selected p38^{JAB1} (JAB1) for further analysis because of its involvement in c-*jun* activation and p27^{Kip1} degradation.

Coprecipitation of PGP9.5 and JAB1 from cell lysates

In order to determine whether PGP9.5 and JAB1 associate *in vivo*, we prepared total lysates from H1299 cells that express both PGP9.5 and JAB1 proteins. Because anti-PGP9.5 polyclonal and monoclonal antibodies could not recognize the non-denatured form of PGP9.5, complexes between endogenous PGP9.5 and JAB1 proteins were precipitated using anti-JAB1 antibody attached to a protein A/G-beads column. The presence of PGP9.5 in the complex was detected by subsequent Western blot using both antibodies. As shown in Figure 1a, PGP9.5 and JAB1 proteins coprecipitated when the JAB1 antibody was used. In contrast, coprecipitation reactions with either an irrelevant antibody (rabbit polyclonal antibody against hemagglutinin, anti-HA) or the protein A/G-beads alone failed to precipitate PGP9.5, indicating that the PGP9.5/JAB1 interaction was specific.

Colocalization of PGP9.5 and JAB1 in vivo

We next examined whether the association between PGP9.5 and JAB1 resulted in colocalization of the two proteins *in vivo* using double immunofluorescence staining of the H1299 cells. As shown in Figure 1b, PGP9.5 and JAB1 were present throughout the cytosol in exponentially growing H1299 cells and JAB1 was also distributed as clusters in the perinuclear space as shown previously (Li *et al*., 2000). Moreover, both PGP9.5 and JAB1 colocalized to the nuclei, where they accumulated as relatively large aggregates. Similar patterns of protein localization were observed in other tested lung cancer cell lines (H157 and A549) (data not shown). To further analyse the nuclear distribution of PGP9.5 and JAB1, an antibody to fibrillarin, a protein that is only present in the nucleolus in interphase cells (Nicol *et al*., 2000) was used together with the JAB1 antibody. The result indicated that PGP9.5 and JAB1

Table 1 Yeast two-hybrid screening using PGP9.5 as a bait

Yeast transformation	*Transformation efficiency*[a] *(cfu/μg library)*	*Transformation yield*[b]	*His+ (1st plate)*	*His+ (2nd plate)*	*Ade+*	*True positives*[c]
1	2.4×10^4	1.2×10^6	488	488	20	8
2	7.0×10^4	3.5×10^6	648	449	38	8
3	2.4×10^5	1.2×10^7	888	690	41	2
Total		1.67×10^7	2,024	1,627	99	18

[a]Transformation efficiency (transformants/μg) = transformation yield ÷ amount of library DNA in μg). [b]Transformation yield (total transformants) = [(colonies/plate) ÷ (volume/plate)] × [(volume of total reaction) ÷ (dilution factor)]. [c]Negative for His and Ade reporter genes after eliminating the Gal4$_{BD}$: bait plasmid

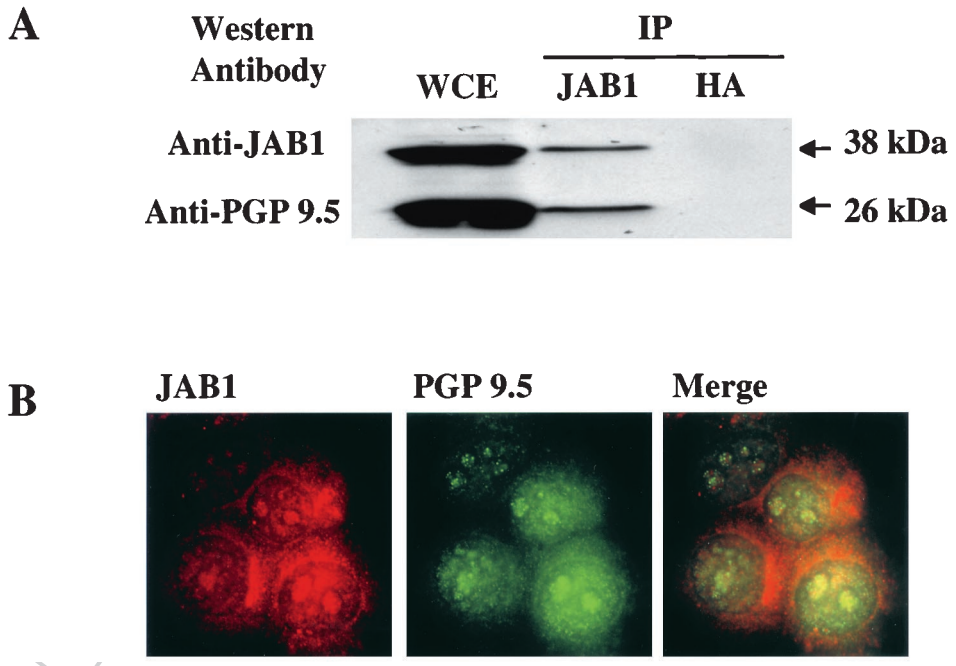

Figure 1 PGP9.5 specifically interacts with JAB1. Endogenous PGP9.5-JAB1 complexes were immunoprecipitated (IP) from H1299 cell lysates with anti-JAB1 antibody and both proteins were detected with anti-JAB1 and anti-PGP9.5 antibodies. Precipitation with anti-HA antibody served as a negative control. WCE: whole cell extract. The sizes of the proteins detected are indicated at the right. (**b**) PGP9.5 colocalizes with JAB1 both to the nucleus and cytoplasm of H1299 cells. Endogenous PGP9.5 and JAB1 were detected by immunofluorescence using anti-p38^{JAB1} and anti-PGP9.5 antibodies and visualized using rhodamine and fluorescein labeled secondary antibodies, respectively. The images were overlapped (Merge) to reveal colocalization

colocalized predominantly to the nucleoli in the nucleus (Figure 2, top panel).

Nucleolar localization and interaction of p27^{Kip1} with JAB1 and PGP9.5

Since JAB1 was known to be involved in the translocation of p27^{Kip1} to the cytoplasm, we tested whether these three proteins colocalized to the same subnuclear compartment using double immunostaining with p27^{Kip1} antibody and either PGP9.5 or JAB1 antibody. As shown in Figure 1b, PGP9.5 and JAB1 were detected both in the cytoplasm and in the nucleoli, whereas p27^{Kip1} was detected primarily in the nucleus with strong staining to the nucleoli (Figure 2). Interestingly, the nuclear localization for p27^{Kip1} was very similar to the colocalization of both PGP9.5 and JAB1. We, therefore, reasoned that these three proteins might be able to form a complex in the nucleoli. H1299 cell lysate used for immunoprecipitation with the JAB1 antibodies and previously immunoblotted with the PGP9.5 antibody (Figure 1a) was separately examined by Western analysis using the anti-p27^{Kip1} antibody. As shown in Figure 3a, p27^{Kip1} protein was also present in the JAB1 complex where PGP9.5 was observed. This *in vivo* interaction between JAB1 and p27^{Kip1} data is consistent with the previous report using *in vitro* synthesized proteins (Tomoda *et al*., 1999). Furthermore, the interaction between PGP9.5 and p27^{Kip1} appeared to be restricted to the nuclear form of the p27^{Kip1} since only K25020 antibody that recognizes mainly the nuclear form of p27^{Kip1} (Figure 3b) was able to coprecipitate with PGP9.5 from the H1299 cell lysate that contained both proteins (Figure 3c). In contrast, PGP9.5 could not be precipitated by this same antibody using the H1155 cell lysate that was devoid of the p27^{Kip1} protein (Figure 3d).

Cellular redistribution of PGP9.5, JAB1 and p27^{Kip1} during serum restimulation

It has been shown that p27^{Kip1} nuclear localization is a prerequisite for it to function as a cell cycle regulator (Polyak *et al*., 1994). JAB1 interacts specifically with p27^{Kip1} and causes its translocation from the nucleus to the cytoplasm, decreasing the amount of p27^{Kip1} in the cell by accelerating its degradation via proteosomes. Our *in vivo* protein localization data suggested that, instead of regulating JAB1 via deubiquitination, PGP9.5 may contribute to the down regulation of p27^{Kip1} by interacting and redistributing JAB1 between the cytoplasm and the nucleus. To test this, we subjected H1299 cells to growth without the serum for 24 h and then examined the cellular distribution of these proteins during serum re-stimulation. As shown in Figure 4, the cellular level of PGP9.5 and JAB1 remained constant while p27^{Kip1} was at its lowest level 4 h after reintroduction of serum into the media. This change of p27^{Kip1} level is also similarly observed by

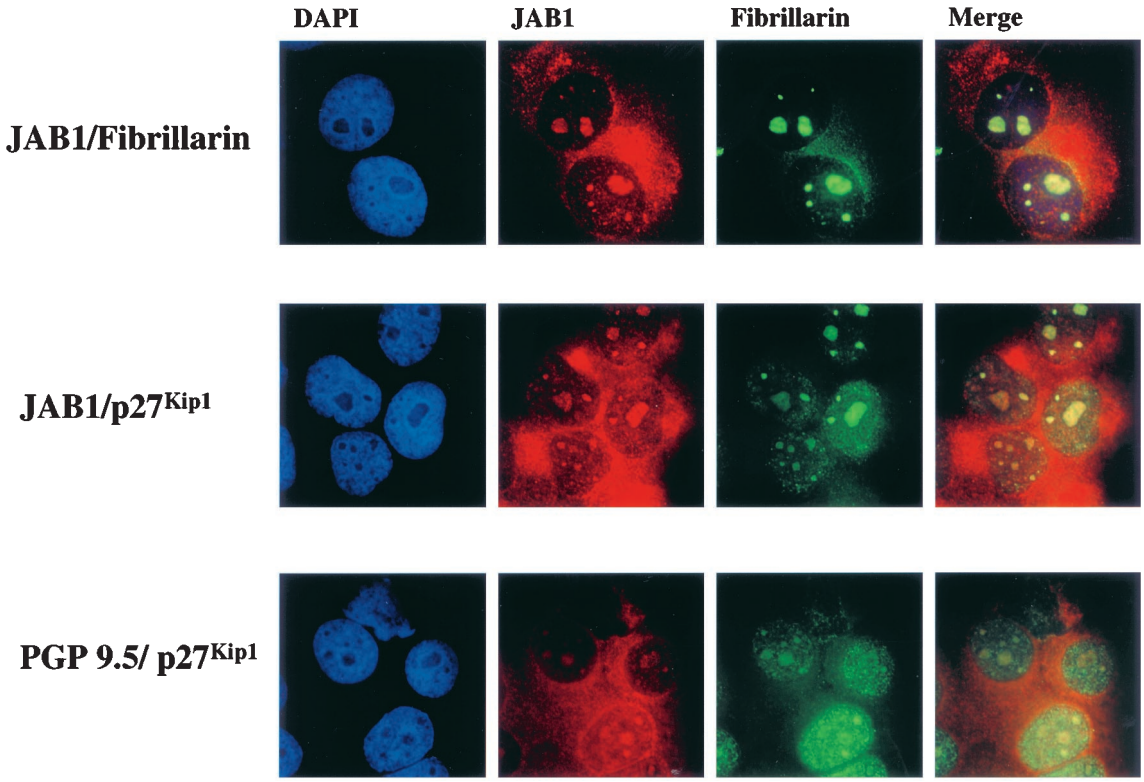

Figure 2 PGP9.5, JAB1, and p27^{Kip1} colocalization in the nucleolus with fibrillarin. Upper panel, H1299 cells were assayed for endogenous JAB1 and fibrillarin localization by immunofluorescence using anti-p38^{JAB1} and anti-fibrillarin antibodies and rhodamine (red) and fluorescein (green) labeled secondary antibodies, respectively. Fibrillarin is a protein that is found in the nucleolus in interphase cells. Middle and lower panels, cellular distribution and co-localization of JAB1, PGP9.5 with p27^{Kip1} in the nucleolus, respectively. H1299 cells were assayed for endogenous, PGP9.5, JAB1 and p27^{Kip1} localization by immunofluorescence using respective antibodies as indicated. Co-localization with p27^{Kip1} was primarily observed in the nucleolus. Nuclei were counterstained with DAPI (blue). The images were overlapped (Merge) to determine co-localization

immunofluorescence analysis shown in Figure 5. At four hours after serum re-stimulation, H1299 cell nucleus was mostly devoid of p27^{Kip1} while PGP9.5 and JAB1 was distributed both in the cytoplasm and the nucleus (Figure 5, top panel). In contrast, hours after serum re-stimulation, when these same cells were allowed to grow to confluency and become contact inhibited, p27^{Kip1} gradually accumulated in the nucleus while PGP9.5 and JAB1 were redistributed to the perinuclear compartment and the cytoplasm of the cells (Figure 5, lower panel).

Discussion

Using the yeast two-hybrid screening, we identified JAB1 as one of the cellular partners for PGP9.5. The JAB1 is a multi-function protein that was first identified by a yeast two-hybrid screen through its ability to associate with the N-terminal c-Jun activation domain. Subsequently, this interaction was shown to potentiate c-jun transactivation and JAB1 was reported to be a nuclear protein (Claret *et al*., 1996). Later, JAB1 was identified as part of a large multiprotein complex named the signalosome (Seeger *et al*., 1998). This complex, which was localized to the cytosol, also contained the mammalian homologues of the plant COP9 complex involved in light-mediated signal transduction (Wei *et al*., 1998). Interestingly, it was shown that JAB1 possesses kinase activity which phosphorylates the N-terminal activation domain of c-*jun*, an essential step for c-*jun* mediated transactivation, as well as IκBα, a regulatory subunit of the NFκB transcription factor (Seeger *et al*., 1998). It was also reported that the macrophage migration inhibition factor (MIF) binds to JAB1 and negatively regulates JAB1-controlled pathways (Kleeman *et al*., 2000).

JAB1 was also shown to interact with p27^{Kip1} in the nucleus, translocate it to the cytoplasm and facilitate its degradation in a 26S proteasome-dependent manner (Tomoda *et al*., 1999). p27^{Kip1} plays a pivotal role in the negative control of cell growth (Polyak *et al*., 1994; Hengst *et al*., 1994). The activity and the cellular level of p27^{Kip1} is mediated through several mechanisms including the inhibition of its binding to CDKs, nuclear sequestration, and degradation via the ubiquitin mediated proteosomes (Slingerland and Pagano, 2000). Clinically, the educed level of p27^{Kip1} protein is frequently observed in human cancers, including breast, lung (Hommura *et al*., 2000), prostate, colon (Yao *et al*., 2000; Hirano and Minamoto, 2000) skin,

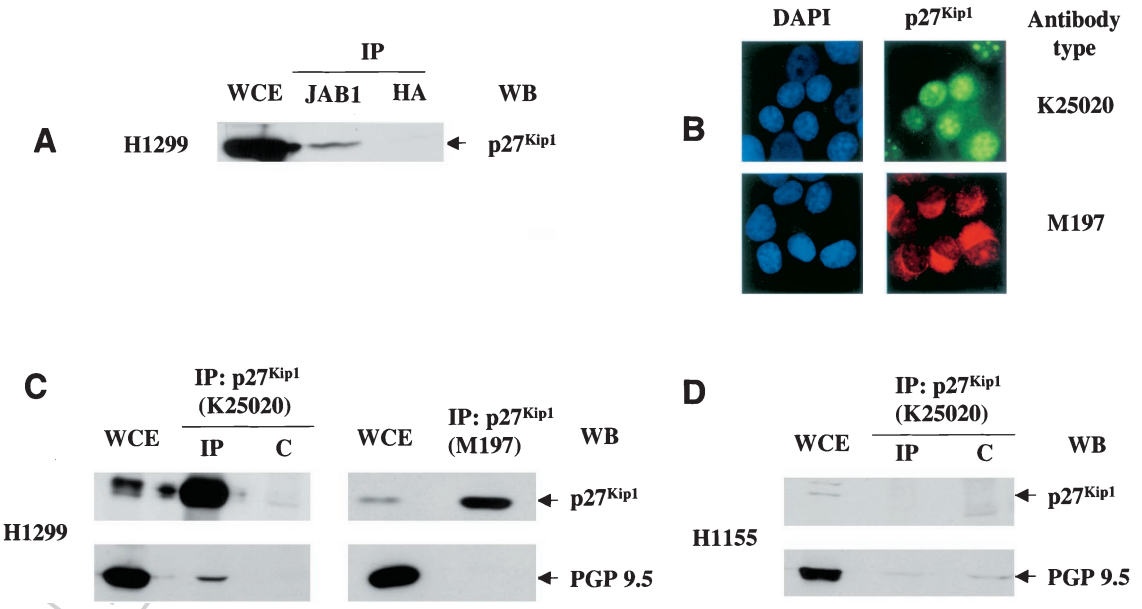

Figure 3 p27^{Kip1} interacts with both PGP9.5 and JAB1. (**a**) H1299 cell lysate used for immunoprecipitation with the JAB1 antibodies and blotted with the PGP9.5 antibody (Figure 1.a) was also blotted separately using the anti-p27^{Kip1} antibody. Endogenous JAB1-p27^{Kip1} complexes were immunoprecipitated from H1299 cell lysate with anti-JAB1 antibody and detected anti-p27^{Kip1} antibody. Precipitation with anti-HA antibody served as a negative control. (**b**) Different anti-p27^{Kip1} antibodies recognize different forms of p27^{Kip1}. Two slides prepared from H1299 collected from the same flask were probed separately with two different anti-p27^{Kip1} antibodies. K25020 recognizes mainly the nuclear form of p27^{Kip1} whereas M197 recognizes mostly the cytoplasmic form of this protein. (**c**) PGP9.5 is in the same complex with p27^{Kip1} in H1299 cell lysate as detected by immunoprecipitation using K25020 anti-p27^{Kip1} (K25020), but not M197 antibody. (**d**) PGP9.5 would not be precipitated from H1155 cell lysate (devoid of 27^{Kip1}) using anti-p27^{Kip1} antibody (K25020). IP: immunoprecipitation. C: Precipitation with beads alone. WCE: whole cell extract. WB: Western blot antibody

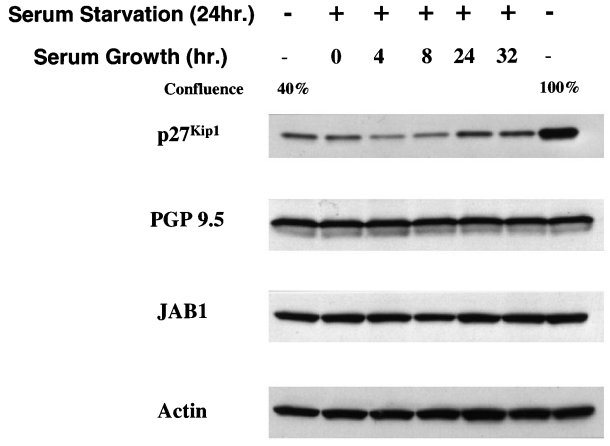

Figure 4 p27^{Kip1}, PGP9.5 and JAB1 levels after serum starvation and re-stimulation. H1299 cells were grown for 24 h in the absence of serum (−) then re-stimulated with serum at 4, 8, 24 and 32 h as indicated before harvest. Levels of p27^{Kip1}, PGP9.5 and JAB1 were compared to H1299 cells grown to 40 and 100% confluence the presence of serum. Actin was used to normalize the amount of protein loaded in the gel

and ovarian cancers. Decreased expression of p27^{Kip1} was shown to correlate with cancer development and poor survival (Slingerland and Pagano, 2000). The exact mechanism that underlies this decreased p27^{Kip1} expression is not known but overexpression of the HER-2/*neu* oncogene was shown to cause mislocation of p27^{Kip1} and JAB1 to the cytoplasm, thereby facilitating p27^{Kip1} degradation (Yang *et al*., 2000).

The relative distribution of JAB1 between the nucleus and the cytoplasm probably reflects the different roles of this protein as a transcriptional coactivator, as part of a cytoplasmic multiprotein complex with kinase activity, and as a factor in p27^{Kip1} translocation. Importantly, the physiological and/or pathological functions of cellular proteins are often reflected by their subcellular localization patterns, particularly in specific subnuclear organelles (Mattson *et al*., 2001). Although JAB1 is not a known substrate of ubiquitination, its colocalization and interaction with PGP9.5 suggests that it may be regulated by PGP9.5 through nuclear transportation. Consistent with this notion is the fact that the functions of the two other interacting proteins, UBC9 and RanBPM, in the yeast two-hybrid assay also suggest that PGP9.5 may indeed participate in protein trafficking between cellular compartments. UBC9 is a ligase for an ubiquitin like protein, SUMO (Sampson *et al*., 2000). SUMO modification is thought to be involved in the nuclear transport of the modified proteins (Lee *et al*., 1998; Melchior, 2000). RanBPM is a newly identified factor that interacts with Ran (Nishitani *et al*., 2001), a small GTP binding protein involved in nuclear traffic of many proteins (Izaurralde *et al*., 1997). Thus, although the exact nature of cellular redistribution of

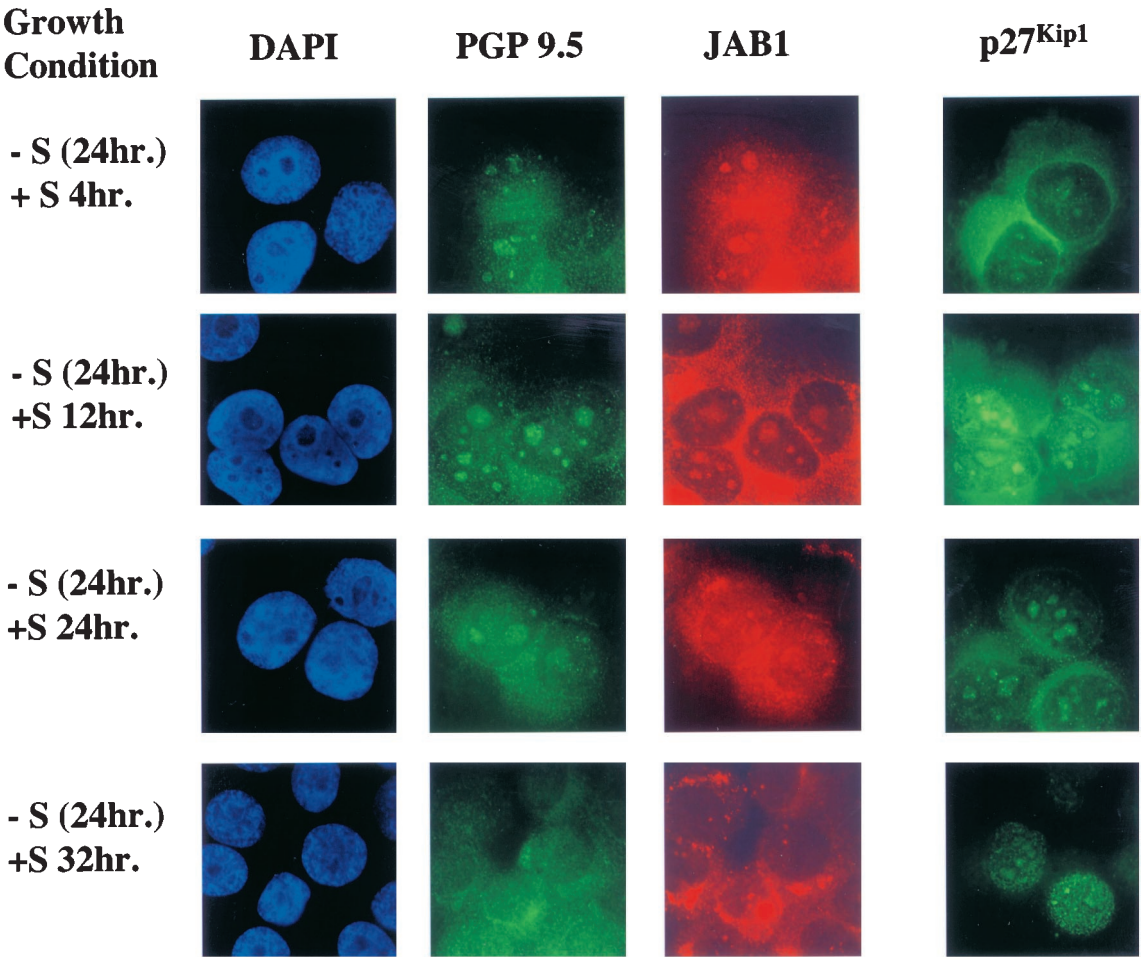

Figure 5 p27^{Kip1}, PGP9.5 and JAB1 localization in H1299 cells during serum re-stimulation. Cells were grown for 24 h in the absence of serum and then examined at 4, 12, 24 and 32 h after serum re-stimulation. H1299 cells were assayed for endogenous PGP9.5, JAB1 and p27^{Kip1} distribution by immunofluorescence using anti-JAB1, anti-PGP9.5, and anti-p27^{Kip1} antibodies and rhodamine and fluorescein labeled secondary antibodies. Nuclei were counterstained with DAPI

JAB1 and PGP9.5 between the nucleus and cytoplasm remains to be determined, the intimate involvement of PGP9.5 with JAB1 and p27^{Kip1} proteins in the nucleus suggests that PGP9.5 may play a role in JAB1 mediated-degradation of p27^{Kip1}. To this end, the high levels of PGP9.5 protein often observed in lung cancers may explain, in part, the observation of low p27^{Kip1} protein levels in including lung cancers.

Materials and methods

Yeast two-hybrid screening

The full length PGP9.5 cDNA (782 bp) was subcloned into the pBD-Gal4Cam vector (Stratagene) containing the yeast *TRP1* marker between the *Sal*I and *Pst*I sites to generate a fusion protein downstream of the Gal4 DNA binding domain (BD). This plasmid was then used as a bait to screen a human fetal brain Matchmaker cDNA library (Clontech), containing the *Leu*2 gene marker (Clontech) and fusion products downstream of the Gal4 activation domain (Gal-AD). The yeast strain PJ69-4A (James *et al*., 1996) was transformed by the lithium acetate method, as described (Gietz *et al*., 1995), with the bait plasmid, then with the Matchmaker cDNA library. The transformants were plated on medium lacking leucine, tryptophan and histidine (−L, −T, −H) for up to 3 weeks at 30°C. Colonies were picked and replated on −L, −T, −H agar plates. Those that grew in the second plate were plated in medium lacking leucine, tryptophan and adenine (−L, −T, −A). The positive colonies were then subjected to several rounds of culture in SC without tryptophan (−T) to eliminate the bait plasmid. Each *Trp*$^{+}$ clone was tested again for activation of the reporter genes to eliminate those that transactivate the reporter gene in the absence of the bait plasmid. Finally, the plasmid DNA was isolated from the positive yeast clones, amplified in *E. coli*, and analysed by automated DNA sequencing. These plasmids were also used to reconstruct the *in vivo* interaction by transforming them back into the original yeast strain containing the bait plasmid.

Cell culture

The lung cancer cell lines H1299, H157 and A549 were maintained in RPMI medium containing 10% fetal bovine serum (FBS), whereas H1155 was maintained in RPMI with

5% FBS. The subcellular localization of the proteins was determined by immunofluorescence staining.

Western blot analysis

Cell extracts were prepared in LSLD buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM DTT, 0.3 mM sodium orthovanadate, 50 mM NaF, 80 μM β-glycerophosphate, 1 mM PMSF and protease inhibitor Complete^{TM} tablet) and subjected to five cycles of sonication (Polyak *et al*., 1994). Fifty microliters of the protein was mixed with an equal volume of 2 × loading buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol), incubated at 95°C for 3 min, and loaded in 4–20% SDS Tris HCl gels (Novex). After electrophoresis, proteins were transferred to nitrocellulose membranes at 280 mA for 1 h. The membrane was blocked by incubation in PBST 5% nonfat dry milk for 1 h, then incubated with the primary antibody overnight at 4°C. After washing in PBST, the membranes were incubated either with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham-Pharmacia-Biotech) for 1 h at room temperature. Antibody binding was detected using the ECL system (Amersham). The antibodies used were polyclonal anti-JAB1 (N-17) from Santa Cruz Biotechnology, monoclonal anti-p27^{Kip1} (K25020, clone 57) from Transduction Laboratories (Lexington, KY, USA), polyclonal anti-p27^{Kip1} (M197) from Santa Cruz Biotechnology, polyclonal and monoclonal anti-PGP9.5 from Biogenesis (New Fields, UK) and monoclonal anti-fibrillarin (AFB01) from Cytoskeleton (Denver, CO, USA).

Co-immunoprecipitations

Polyclonal anti-JAB1 antibody was cross-linked to protein A/G beads using the Immunopure IgG plus orientation kit (Pierce) according to the manufacturer instructions. Four milligrams of H1299 protein extract, prepared with LSLD buffer, was applied to the column and, after 1 h incubation at 4°C, the column was washed with binding buffer. The bound protein was eluted and the fractions with higher protein content were collected and loaded in a 4–20% SDS Tris-HCl gel. Columns without bound antibody or with an irrelevant polyclonal antibody (rabbit anti-HA, Pierce) were used under the same conditions to serve as negative controls. For p27^{Kip1} immunoprecipitation, H1299 or H1155 cell lysates (4 mg total protein), prepared in LSLD buffer, were incubated with the antibody for 2 h at 4°C with gentle agitation. Immunocomplexes bound to protein A/G beads were collected by centrifugation and washed several times in LSLD buffer. Immunoprecipitated proteins were resolved in 4–20% SDS–PAGE gels followed by Western blot.

Immunofluorescence staining

Cells were harvested by trypsinization, washed with PBS, and cytospun onto the microscope slides. Cells were fixed with 3% paraformaldehyde in PBS (138 mM NaCl, 2.7 mM KCl, pH 7.4) for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 2 min and blocked with 5% goat serum in PBST (PBS 0.1% Tween 20) for 30 min at room temperature. Cells were then incubated with the primary antibody overnight at 4°C and, after three washes in PBST, were incubated with a mixture of secondary antibodies (FITC-conjugated goat anti-mouse IgG and Rhodamine-conjugated goat anti-rabbit IgG, Santa Cruz Biotechnology). After washing, the slides were incubated with 0.02 μg/ml DAPI (Sigma) to visualize the nuclei. The slides were mounted in Vectashield and analysed under a Zeiss Auxiphot epifluorescence microscope.

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